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# Studies in Bacterial Metabolism



**STUDIES IN BACTERIAL METABOLISM WITH  
SPECIAL REFERENCE TO CREATINE  
AND CREATININE**

BY

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**THESIS**

FOR THE

**DEGREE OF BACHELOR OF SCIENCE**

IN

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

RUDOLPH ALFRED FAUST

ENTITLED STUDIES IN BACTERIAL METABOLISM WITH SPECIAL REFERENCE

TO CREATINE AND CREATININE

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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STUDIES IN BACTERIAL METABOLISM WITH  
SPECIAL REFERENCE TO CREATINE  
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INTRODUCTION.

Creatinine, an end product of nitrogenous metabolism, has usually been considered as being of animal origin, in fact, only the vertebrates were considered as capable of synthesizing it in their metabolism. Creatine, the mother substance of creatinine, has thus far been isolated only from vertebrate muscle.

Yet Sears, (1) and German (2) claimed to have obtained color reactions indicating its presence in various bacterial cultures and have interpreted the result to mean that bacteria, a form of vegetable life distinctly different in their functions from animal life, were capable of producing creatinine as a product of nitrogenous metabolism. This similarity, if true, would lend a remarkable analogy between these two forms of life. None of the investigators, however, who report the presence of creatinine in bacterial cultures were able to isolate creatinine or creatine.

In as much as the color reactions of creatinine are also given by many other substances, the problem invited further investigation to determine more conclusively







whether creatinine really was formed in bacterial cultures.

### Review of Literature

Several bacteriological investigators, namely; Sears(1), German (2), Antonoff (3) and several others have worked on this problem and have accepted color tests as positive evidence of the presence of creatinine. The two standard color tests used were Jaffe's picric acid and Weyl's sodium-nitro-prusside test.

Zinno (5) succeeded in precipitating creatinine as creatinine-zinc chloride from several species, excluding, however, bacillus typhosus.

Fitzgerald and Schmidt (4) using glucose free media got negative results for the presence of creatinine in nearly all cases and concluded that certain bacteria either produce no creatinine at all, or produce it in amounts too small to be detected. These investigators were unable to parallel German's results using the same micro-organisms. Their methods, in general, were of the same nature, Sears' being perhaps the most representative. He used a Witte's peptone-glucose medium inoculated with numerous species of bacteria, with incubation for periods of 5-7 days at 37°C. This medium was composed of 2% Witte's peptone, 1% glucose and 0.5% sodium chloride. 100 cc of this solution were placed in large Erlenmeyer flasks and 5 grams of solid calcium carbonate were



added to each ( to counteract any acidity developed). These flasks were then sterilized, inoculated and incubated. After 7 days the cultures were autoclaved and the resulting end products of bacterial metabolism studied. He gives no explanation of his methods of analysis other than to state that he used the Jaffe reaction for qualitative tests and the Folin method for its quantitative estimation. Of the numerous species of bacteria tested, Sears found that bacillus typhosus yielded the largest amounts of Creatinine.

The methods employed by other investigators were very much the same, that is, all of them determined creatinine by color tests but none of them had ever isolated it as such or as a complex salt.

### EXPERIMENTAL PART

The color produced in the Jaffe test is due to the reduction of picric acid in alkaline solution to the red sodium salt of picramic acid and since the test is essentially a reduction test, there is the possibility that many other substances beside creatinine give the same result. Glucose, in hot solution and acetone in the cold, both give the red coloration that is given by creatinine.

Hence, it would be desirable, if a conclusive test for creatinine were to be made, to isolate it in some form and then apply the picric acid and alkali test for color changes.





Thus any reducing agents that might be present when a test was applied in solution, would be eliminated. If it could be isolated from bacterial culture, then the work of the bacteriological investigators could be substantiated more definitely, and with this aim in view, this investigation was started.

Unfortunately, creatinine does not form many insoluble salts. The creatinine zinc-chloride and the potassium creatinine-picrate are the only two that offer any encouragement for the possibility for precipitation.

Before proceeding with any precipitation methods, however, interfering substances had to be removed. Of these, the peptones were the ones that had to be taken out of solution. Precipitation by alcohol proved to be the best after the use of alumina cream and colloidal iron had failed to be satisfactory. Four volumes of 95% alcohol added to the medium as above prepared precipitated nearly all the peptones.

#### Removal of Peptones by Alcohol.

500 cc of the peptone medium to which was added a small amount of KCl was prepared. This was divided into two parts; one for a blank or "control", the other for experimental precipitation.

#### Part I

To one part I added 50 mgs. of pure creatinine prepared by Benedict's method (6) and reduced the volume to 25cc on the steam bath. To this I added four times its volume of



95% alcohol. On standing over night a heavy white precipitate settled out. The filtrate from this was taken down to 75cc and then 3 grams of picric acid in 15cc of alcohol added. This was placed in the cold over night and a yellow amorphous precipitate came down which was supposedly potassium-creatinine-picrate. This precipitate was redissolved in hot alcohol and placed in the ice box. The substance was recrystallized and gave a good Jaffe test. The crystals had no definite structure and were probably not a definite compound.

## Part II

The second portion which was to serve as a control was treated in a similar manner except that no creatinine was added. On standing a crystalline precipitate formed in this case also, yellow in color and having a rosette structure. It, however, gave no color test for creatinine. The precipitate was probably a picrate complex with the amino acids from the peptones.

These results were encouraging, since they suggested a method for identifying creatinine in a peptone medium. If then, this crude precipitate could be purified and comparatively pure potassium-creatinine picrate obtained the method applied to bacterial cultures would solve the problem. The following is the experimental method used for purification.

Acetone was used as a solvent for potassium-creatinine picrate. A mixture of this and free picric acid, when shaken up and extracted with acetone should be freed from





the creatinine salt which is taken out with the solvent. For the crude picrate I used the precipitate obtained on adding alcoholic picric acid to urine as described by Benedict (6). This precipitate is a mixture of potassium creatinine picrate, free picric acid and urinary salts. From 1.63 grams of this crude material, 1.0465 grams were extracted by acetone. This extracted portion ( after evaporation) was dissolved in about 50cc of water, which after long standing gave .2472 grams of a precipitate of long brown needles. These were assayed for their purity in a Duboscq colorimeter by Folins Microchemical Method (7).

#### Experiment:

0.1113 grams of crude potassium creatinine picrate were dissolved in 20 cc of water and 1cc of the solution taken for analysis. This was compared with a 1 mg. standard of pure creatinine as detailed below.

Reading of Standard	Reading of Unknown
9.53	8.3
13.34	11.2
14.29	12.3

Creatinine in 1 cc (theoretically)	.00165 grams
" " " ( found )	.00114 "

Repeated analysis showed that the potassium-creatinine-picrate could be obtained by this method 55-70% pure.



### Preparation of Creatinine-Zinc-Chloride. .

About one half gram of the crude picrate material was dissolved in 75cc of water to which had been added 10 drops of concentrated sulfuric acid. The acid was for the purpose of liberating the picric acid so that in the subsequent extraction with benzene, the picric acid could be removed, leaving the creatinine as such in solution. Then repeated extractions with benzene were made, the color of the solution becoming paler with each extraction, showing that the picric acid was being removed. The purified solution was then neutralized with an excess of magnesium oxide, filtered and to it added 10cc of zinc chloride and glacial acetic acid. When then four volumes of alcohol were added a heavy precipitate came down. This was probably magnesium sulphate. It was filtered off and the filtrate allowed to stand four days in the cold. No precipitate of creatinine-zinc-chloride was formed.

While this method of precipitation of creatinine is applicable for its isolation, it cannot be used when small quantities are dealt with. Consequently the method was abandoned in favor of the picrate precipitation.

### Precipitation of Peptones by Tungstic Acid

Folin (8) in his system of blood analysis used equal volumes of 10% sodium tungstate and 2/3 normal sulfuric acid to precipitate the proteins in preparation of blood filtrates. This





method applied to peptone media was found to work very satisfactorily and proved to be a better precipitant than alcohol alone.

I prepared a peptone-glucose medium as described above and to a 100cc portion ( reduced to 50cc by distillation) added 10cc of a 10% solution of sodium tungstate and 10cc of two thirds normal sulfuric acid. A heavy precipitate of peptones came down but on adding more sulfuric acid a further precipitation occurred. On experimenting it was found that 15-20 cc of this strength acid was necessary for the complete removal of the peptones.

The results of the above experiments now offer a method of determining creatinine in peptone cultures as used by Sears, namely;

1. Precipitation of the peptones by tungstic acid.
2. Precipitation of the creatinine as the double salt of potassium-creatinine-picrate by picric acid.
3. Purification of the precipitated salt by acetone.

#### Experiments with Bacterial Cultures.

A litre of a solution containing 2% Wittes peptone, 1% of glucose and 0.5% sodium chloride was prepared. To each of ten three litre Erlenmeyer flasks were added 100cc of this solution and five grams of solid calcium carbonate. The tops were then loosely plugged with cotton, the flasks sterilized and finally inoculated with bacillus typhosus. All flasks were placed in an electric incubator and kept at 37°C for seven days.





Bacteriological examination made at this time by Professor F.W. Tanner showed the presence of large numbers of living organism. At the end of this period they were autoclaved to destroy the organism and the calcium carbonate filtered off. Each 100cc portion was then reduced on volume by vacuum distillation to about 30cc. This treatment was followed by the precipitation of the protein by tungstic acid, using the quantities of reagents described above. The clear filtrates were again reduced in volume by distillation and then combined into one with the addition of a little sodium acetate to neutralize the excess sulfuric acid. This combined filtrate was evaporated to about 50cc and poured into four volumes of 95% alcohol. A light precipitate settled out which was the remaining peptones. The alcohol was evaporated from the filtrate and then 10cc of freshly prepared saturated picric acid added. No precipitate formed and the brownish-red solution was taken down over a steam bath until a gummy mass remained. To prevent scorching by further heating, air was blown over the mass to complete the drying. The residue, however, would not dry completely. The mass in this condition was now ready for extraction.

Extraction:- About 60cc of acetone were shaken with the residue in an Erlenmeyer flask. The acetone became brownish yellow. Several such extractions were made and it was assumed that both the excess picric acid and the creatinine-picrate were thus removed.

The acetone solution was now evaporated and the resi-



due treated with benzene which would remove the picric acid but leave the creatinine salt. The residual mass, a brown substance, was now ready for analysis.

Color Test:- I took a small portion of the residue and added about 5cc ether and evaporated. This was to assure the removal of all acetone since acetone will respond to the Jaffe test. After evaporation no color was produced on adding picric acid and sodium hydroxide; namely; test for creatinine was negative.

Precipitation:- The remaining portion was dissolved in the minimum amount of water and a crystal of KCL added for the purpose of forming the potassium-creatinine complex. However, no precipitate was formed upon standing over night in the cold. On evaporation the residue pointed to the absence of creatinine.

Control Test:- The control test was conducted by going thru the same procedure as above, omitting the bacteria inoculation and subsequent incubation. Acetone extractions from the gummy residue was made and the same test for creatinine applied as in the above experiment. All tests were negative.

I noted, however, that the Jaffe test brought about a very slow change in color, especially on standing over night. This was noticed in both the bacterial cultures and in the control. The color was not the distinct red creatinine color, but a brownish-red shade. I concluded, from the fact that it appeared in the control as well as in the culture and also from





its slowness of formation, that it was not due to creatinine, but probably to the glucose.

Recovery of Added Creatinine from Media:- To determine whether creatinine could be recovered when added to a medium, I dissolved 30 mgs. in 100cc of the peptone preparation and carried out the procedure in identically the same manner as in previous determinations. A portion of the reddish-brown residue from the acetone extraction was dissolved in a small amount of water in a test tube. The addition of picric acid turned it yellow but a sharp change to red was noted when the alkali was mixed with it. This color change was sharp and distinct and pointed decisively toward the presence of creatinine. The remaining portion of the residue was taken up in water in a small dish and a crystal of potassium chloride was dissolved in it. It was placed in the cold and allowed to stand over night, but no precipitate formed.

Although the color test for creatinine was positive, attempts to isolate it were unsuccessful.

## DISCUSSION

In these experiments I have shown that it is possible by these methods to carry any appreciable amounts of creatinine through to the final steps for analysis in a glucose-peptone medium and get positive results for its presence by the Jaffe color test. It could not, however, in any case be precip-



tated and isolated as the potassium-creatinine-picrate or the creatinine-zinc-chloride.

Bacterial cultures of bacillus typhosus when prepared and analyzed by the foregoing methods, failed to give any indication of the presence of creatinine. Tests on the control media gave no color reactions.

The conclusions drawn from this work are that the typhoid bacillus does not form creatinine under the present experimental conditions. These conditions were the same as those under which Sears (1) obtained a maximum production of creatinine. This investigator found that creatinine (?) production by bacillus typhosus, the same organism used in the present study was so constant that this property was proposed for the differentiation of bacillus coli and bacillus typhosus.





## SUMMARY

From cultures of bacillus typhosus in a medium of Wittes' peptone, glucose, sodium chloride and calcium carbonate, we were unable to isolate creatinine, either as the potassium-creatinine-picrate or the creatinine-zinc-chloride nor to obtain the Jaffe color test.

Control test made on the same media to which small amounts of (30-50 mgs.) creatinine had been added, showed strongly positive Jaffe' reactions, but no creatinine could be isolated as the double potassium-creatinine-picrate or as creatinine-zinc-chloride.

These results indicate that for the bacillus typhosus which has been said to form creatinine under the present experimental conditions, creatinine is not a product of nitrogen metabolism.



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